NUMBERS OF ENTEROCOCCI IN WATER, SEWAGE, AND FECES DETERMINED BY THE MEMBRANE FILTER TECHNIQUE WITH AN IMPROVED MEDIUM[1]

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In a previous report from this laboratory (Slanetz *et al.*, 1955), a highly selective medium was described for use with membrane filters in the enumeration of enterococci or fecal streptococci in water. Higher counts for enterococci were generally obtained with this procedure than with other methods described. The technique afforded a relatively simple and direct means for the determination of numbers of enterococci in water, sewage, or other materials.

After studies of the effects of various amounts and combinations of peptones and other ingredients in media for the cultivation of enterococci on the membrane filters, it became evident that improvements could be made. As a result, a new medium has been formulated. The composition and efficiency of this medium are described.

MATERIALS AND METHODS

Bac-T-Flex type membrane filters (S & S—Schleicher & Schuell Co., Keene, N. H.) were used for most of our studies. The filters were placed between absorbent pads, wrapped in Kraft wrapping paper, and autoclaved at 121 C for 10 min.

Samples of feces, sewage, and polluted water were used as sources of enterococci for our studies. With a vacuum pump, the desired amounts and dilutions of the samples were filtered and the filter funnel rinsed with about 30 ml of sterile buffer solution. A Coli 5 filter apparatus (S & S) with a 30 ml capacity funnel was found most convenient for filtration. After filtration, the filters were transferred directly to the surface of the agar medium in 60 mm diameter petri dishes. The cultures were incubated at 35 C for 48 hr under normal conditions of humidity. Colony counts were made with a stereomicroscope magnifying 10 times.

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RESULTS

In studying various amounts and combinations of peptones in media used for the cultivation of enterococci on membrane filters, certain preparations containing trypticase (Baltimore Biological Laboratory) and phytone (BBL) produced higher enterococcus counts than the enterococcus medium described by Slanetz *et al.* (1955). However, the colonies of enterococci were smaller and less distinct. These results indicated that in some samples there were probably enterococci of lowered viability that were unable to grow satisfactorily on the membranes placed on pads saturated with our selective media. As suggested by Allen *et al.* (1953) and Childs and Allen (1953) this may have been due to the age or prolonged immersion of the organisms in water.

Further studies to improve the composition of our enterococcus medium resulted in the formulation of a new medium hereafter referred to as the M-Enterococcus Agar. This medium contains 2 per cent tryptose (Difco), 0.5 per cent yeast extract, 0.2 per cent glucose, 0.4 per cent dipotassium phosphate, 0.04 per cent sodium azide, 0.01 per cent 2,3,5-triphenyl tetrazolium chloride (TTC), and 1 per cent agar. In the preparation of this medium, the first five ingredients listed are dissolved in the appropriate amount of distilled water and the pH adjusted to 7.2. The agar is then added and the solution heated sufficiently to dissolve the agar. After it is cooled slightly, 1 ml of a 1 per cent sterile solution of TTC is added per each 100 ml of medium. The medium is then poured directly into petri dishes.

In our hands, the above medium has given better results for the enumeration of enterococci than any of the other media tested to date. Incubation of the filters directly on the agar surface resulted in larger sized colonies and higher counts of enterococci than were obtained when the filters were incubated on pads saturated with the liquid medium. The medium has proved 100

per cent selective for fecal streptococci even when heavily polluted water samples were filtered through the membranes. For determining the selectivity of the medium, all the colonies developing on the filters for many of the samples were isolated, and over 2500 such isolations have all been identified as fecal streptococci. As noted previously, it is not necessary to sterilize this medium other than by boiling the agar; in fact, autoclaving with steam under pressure reduced the efficiency of the medium. This is particularly true for the tellurite sensitive streptococci, such as *Streptococcus durans*, *Streptococcus faecium* (Oral-Jensen), and *Streptococcus bovis*. With this medium, the fecal streptococci appear on the filters as pink to dark maroon colored colonies from 0.5 to 3 mm in diameter depending on the species or types present in the sample being tested. The size and appearance of the colonies are shown in figure 1. It should be noted that the term *enterococcus* as used in this paper refers to all types of streptococci commonly inhabiting the intestinal tract of man and animals. This would include such streptococcus organisms as *S. bovis*, and *S. equinus* as well as *S. faecalis*, *S. liquefaciens*, *S. zymogenes*, and *S. durans*. No growth was obtained on the filters with this medium when suspensions of the following organisms were tested: *Escherichia coli*, *Aerobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *Sarcena lutea*, *Bacillus subtilis*, and *Bacillus cereus*.

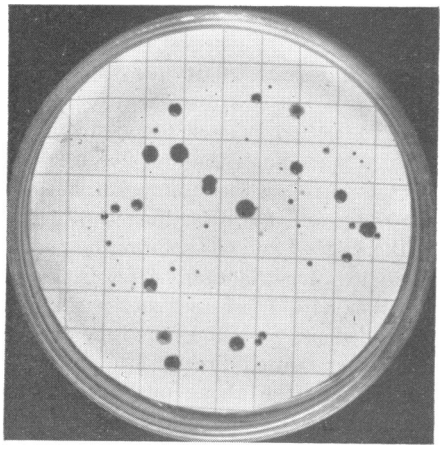

Figure 1. Colonies of enterococci on a 50 mm diameter Bac-T-Flex filter when incubated on the enterococcus medium; 100 ml of polluted river water were filtered through the membrane; all other types of bacteria in the water have been inhibited; no magnification.

Since it would be desirable to have the enterococcus medium available in a dehydrated form, experimental lots of this medium were supplied to us by Difco Laboratories with and without TTC added. A dehydrated preparation without TTC was also supplied by Baltimore Biological Laboratories in which 1.5 per cent trypticase (BBL) and 0.5 per cent phytone (BBL) were substituted for the tryptose (Difco). The dehydrated enterococcus medium (Difco) gave approximately the same counts for enterococci as the medium prepared from the separate ingredients in our laboratory. While somewhat lower counts of enterococci were obtained with the dehydrated medium (BBL) for the samples tested, this medium also appeared satisfactory for enterococcus determinations.

Comparative numbers of enterococci and coliforms in water as determined by membrane filter and Most Probable Number (MPN) techniques. With the improved M-Enterococcus Agar, the efficiency of the membrane filter technique for the enumeration of enterococci in water was compared to the MPN procedure described by Litsky *et al.* (1953*a*). For this procedure, dextrose azide broth (Difco) was used as the presumptive medium and ethyl violet azide broth (Difco) as the confirming medium. For the determination of numbers of coliform bacteria, the filters were incubated directly on absorbent pads saturated with BBL-M-Endo broth as outlined by Slanetz and Bartley (1955). For the enterococcus and coliform MPN determinations, portions of samples inoculated were five 10-ml, five 1-ml, and five 0.1-ml amounts. The MPN index of coliforms was determined according to *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes* (American Public Health Assoc., 1955) with lactose broth and confirmation in brilliant green lactose bile broth.

The results obtained for tests on various water samples are recorded in table 1. Both the arithmetic and geometric mean counts are given for comparative purposes. In general, higher counts of enterococci were obtained by the membrane filter technique than by the MPN procedure, the mean ratio being 1.9:1. The mean ratio of enterococci to coliform bacteria for the samples tested by membrane filter techniques was also

TABLE 1

Numbers of enterococci and coliforms in polluted water as determined by membrane filter techniques

Samples	Enterococci		Coliforms	
	Membrane filter technique	MPN technique	Membrane filter technique	Std 5-tube MPN technique
River a	226*	234	60	22
River b	135	41	182	182
River c	36	41	11	18
River d	1,095	138	593	270
Brook	215	182	103	176
Reservoir	415	247	42	131
Well a	85	20	35	110
Well b	8	17	8	46
Well c	124	8	260	540
Well d	539	540	193	17
Well e	26	23	0	0
Well f	10	13	0	2
Arithmetic mean.	242.8	125.3	123.9	126.2
Geometric mean..	102.0	56.6	35.7	43.2

* The numbers given for rivers, brook, and reservoir represent the mean of 2 to 8 determinations on samples from each source per 100 ml of water.

1.9:1. It is interesting to note that for this series of tests, the membrane filter counts for coliforms correlated very closely with the MPN determinations for these organisms. It should be pointed out that the above ratios are based on the numbers of the coliform group of bacteria and not numbers of *E. coli*. For example, of 603 strains of coliforms isolated from various water samples, only 46.6 per cent was identified as *E. coli*. Thus, the ratio of enterococci to *E. coli* would be appreciably higher. Litsky *et al.* (1955) reported a final mean ratio of enterococci to *E. coli* as 7.6:1 for water samples from the Connecticut River.

Comparative numbers of enterococci and coliforms in samples of sewage and feces as determined by the membrane filter technique.[2] The numbers of enterococci compared to the numbers of coliform bacteria in sewage as determined by the membrane filter technique are listed in table 2. The samples of sewage were collected at the Durham sewage treatment plant at different periods. While there was considerable variation in the numbers of enterococci and coliforms in the different samples, the arithmetic mean ratio for 25 samples tested was 1 enterococcus to 1.7 coliforms, and the geometric mean for these samples was 1 enterococcus to 1.5 coliforms. It should again be emphasized that this ratio is based on the coliform group and not *E. coli*. Thus, out of 426 strains of coliforms isolated from some of these sewage samples, only 57 per cent were identified as *E. coli*. It is probable that many of the other types of coliforms developing on the filters were of nonfecal origin. The density of enterococci as compared to coliform bacteria in the sewage samples reported above was the greatest of any reported thus far in the literature. With the MPN procedure, Litsky *et al.* (1953*b*) reported the density of coliform bacteria to be approximately 13.3 times that of enterococci in the samples of raw sewage tested.

The numbers of enterococci and coliforms as

TABLE 2

Numbers of enterococci and coliforms per milliliter of sewage sample as determined by membrane filter techniques

Sample	Enterococci	Coliforms
1	5,495	35,400
2	4,960	5,280
3	3,930	1,235
4	24,850	6,230
5	15,400	2,270
6	11,400	21,000
7	4,025	650
8	1,340	2,800
9	625	387
10	1,310	625
11	4,776	38,600
12	4,500	12,500
13	13,100	34,500
14	260	150
15	1,300	840
16	300	3,500
17	2,040	570
18	210	870
19	10	100
20	725	2,405
21	190	4,050
22	1,280	250
23	20	760
24	3,357	7,100
25	1,892	4,700
Final arithmetic mean.....	4,295.8	7,470.9
Final geometric mean.....	1,413.8	2,255.6

[2] Some of the data reported in tables 2 and 3 were taken from a thesis (Sullivan, 1956).

TABLE 3

Number of enterococci and coliforms in feces from human beings and animals as determined by membrane filter techniques

Samples	Enterococci	Coliforms	Samples	Enterococci	Coliforms
Human	9,800*	3,375	Bovine	276	198
	5	20,000		3,550	299
	310	41,050		73	360
	10,000	14,750		1,010	180
	83	10		76	2
	2	292		155	1
	500	740		387	1
	238,000	8,800		10	3
	390	1,000		22	700
	552	375		5	1,200
	205	3,500		13,500	59
	18,000	1,640		450	5
	170	100		3,287	406
	16,000	5,460		1,795	258
	886	186		191	650
	3,900	6,490	Sheep	910	450
	9	251		347	1,490
	5,310	3,340	Horse	150	14
	270,000	258,000	Dog	392,000	55,000
	27,150	10,000		500,000	8
Arithemitic mean	30,063.6	18,968.0	*Arithmetic mean*	45,909.7	3,064.2
Geometric mean	1,108.5	2,083.9	*Geometric mean*	586.3	91.3

* Numbers indicate enterococci and coliforms in thousands per gram of feces.

determined by membrane filter tests in samples of feces from man and animals are given in table 3. As compared to previous data available in the literature, surprisingly large numbers of enterococci were found in many of the fecal specimens. For 20 samples of feces from human subjects, the arithmetic mean ratio of enterococci to coliforms was 1.6:1, and the geometric mean ratio was 1:1.7. For 20 samples of feces from different animals, the arithmetic mean ratio of enterococci to coliforms was 14.9:1, and the geometric mean ratio was 6.4:1. As can be noted in table 3, there was considerable variation in the numbers of these bacteria in different subjects, and we also noted considerable variation in the numbers of these enteric organisms in samples from the same subject taken on different days. While little information is available in the literature on the numbers of enterococci in human and animal feces, the numbers found to be present in our studies with the membrane filter technique and the M-Enterococcus Agar are the highest ever reported. Houston (1932) reported the examination of 14 mixtures of 10 human stools each and found one mixture to contain 10,000 streptococci per g, another mixture 10 million per g, and the other mixtures 100,000 to 1 million per g. Winter and Sandholzer (1946) found an average enterococcus count of 140,000 per g for nine samples of human feces, which was approximately one-hundredth of the average coliform count for these samples. As Litsky *et al.* (1955) have pointed out, the low numbers of enterococci recovered from various sources by previous methods was probably due to the failure of the media employed to detect these organisms.

DISCUSSION

The results obtained during this study confirm the conclusions of Slanetz *et al.* (1955) that the membrane filter technique affords a direct and efficient procedure for the detection and enumeration of enterococci in poluted water or other materials. The development of an improved medium for use with the filters has further increased the accuracy and usefulness of this procedure. While the techniques for the enumeration of enterococci in water described by Litsky *et al.*

(1953*a*) gave comparable results for many of the samples tested, the membrane filter technique afforded direct counts of enterococci in a 48-hr period. This method is simpler to perform and requires no confirmation tests. To date, the enterococcus medium has proved 100 per cent selective for enterococci when it is used with membrane filters for samples of polluted water, sewage, or feces. The colonies of enterococci do not change in appearance other than possibly to increase in size when held at room temperature after the original 48-hr incubation period. Thus, when necessary, counts may be made after 48 hr, or the filters may be kept for future reference.

A large number of strains of enterococci have been isolated and identified from the membranes used for tests on water, sewage, or fecal samples. On the basis of the results obtained to date, it is evident that all species and varieties of fecal streptococci will grow on the filters incubated on the selective medium described.

SUMMARY

An improved medium for use with membrane filters for the enumeration of enterococci in water and other materials is described. This membrane filter procedure affords direct counts of enterococci on the filters and takes less time and effort than any other techniques so far described for the detection of these organisms. Since the medium appears to be 100 per cent selective for enterococci, no special skill is necessary for differentiating colonies on the filters; all colonies present are enterococci or fecal streptococci.

Based on the arithmetic mean counts of all samples tested during this study, the ratio of enterococci to coliforms was 1.9:1 for the water samples, 1:1.7 for the sewage samples, 1:1.6 for fecal samples from human beings, and 15:1 for fecal samples from animals. These are the largest ratios of enterococci to coliforms so far reported for such samples. With this membrane filter technique, it would appear that the detection of enterococci will prove to be the most efficient and accurate method so far available for determining the sanitary quality of water or other materials.

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